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Expression of leukocyte antigen CD34 by brain capillaries in Alzheimer's disease and neurologically normal subjects*

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Summary. We studied the expression of a hemopoietic progenitor cell antigen, designated CD34, in brains from subjects with Alzheimer's disease (AD) and non-neurological controls. Immunoblots of brain microvessel proteins probed with monoclonal antibody QBEND/10 to the leukocyte antigen CD34 recognized a protein band with an apparent molecular mass of 90–100 kDa. Immunocytochemical staining of brain tissue sections showed CD34 to be expressed by all microvasculature including those of the circumventricular organs. In normal control brains such specific staining exhibited by QBEND/10 was indistinguishable from that obtained with collagen IV antibodies. In AD, however, increased vascular tendrils in form of endothelial abluminal processes and intraparenchymal abnormalities were evident in cortical and hippocampal regions, predominant in cases with severe pathology. Our results demonstrate that the leukocyte antigen CD34 is localized with the vascular endothelium throughout the human brain. These results also suggest that CD34 detects endothelial abnormalities in brains of AD subjects and support previous observations on the usefulness of CD34 to label abluminal microprocesses.

Key words: Alzheimer's disease – Brain endothelium – Blood-brain barrier – Hemopoietic progenitor cells – Leukocyte antigen CD34

The human hemopoietic progenitor cell antigen CD34 has recently been described to be associated with cells in the hemopoietic lineage [2, 3, 19]. However, monoclonal antibodies (mAb) to CD34, ICH3 and QBEND/10 also recognize proteins or epitopes in the microvasculature of normal [19] and tumor tissues [3, 18]. Schlingemann et al. [18] recently suggested that CD34 may be useful as an endothelial marker for angiogenesis. They noted predominant luminal immunostaining of the endothelium in normal resting tissue and observed striking staining of endothelial abluminal microprocesses (EAM) in tumor stroma, which were observed both at tips of vascular sprouts and on fully formed microvessels [18].

We recently observed that in brain tissue of some subjects with Alzheimer's disease (AD) there were increased vascular peculiarities stained by anti-collagen IV sera ([12, 13] and Kalaria, unpublished observations). McGeer et al. [16] reported similar observations and found an increased proportion of 'strings' or 'streamers' in brain capillaries stained by antibodies to laminin and collagen IV in subjects with AD and other neurological diseases. In related vascular studies, we further found evidence for increased transferrin receptor, a marker for proliferating cells, in cerebral microvessels from some AD subjects [14]. Taken together, these observations suggest that vascular proliferation or degeneration analogous to reactive gliosis exists in chronic neurological conditions such as AD. We therefore compared patterns of immunostaining in human brain tissue between normal control and AD subjects using an mAb to the leukocyte antigen CD34 as a potential marker for angiogenic activity [18]. We also compared the regional distribution of CD34 expression to that of collagen IV and Factor VIII immunostaining in brain regions of neurologically normal subjects.

Materials and methods

Materials

The mAb QBEND/10 to leukocyte antigen CD34 was purchased from Serotec, UK. Antibodies to collagen IV, Factor VIII and transferrin were purchased from Chemicon Inc., and the DAKO corporation. Brain tissue was obtained from the autopsy service division of University Hospitals Cleveland and the Cuyahoga County Medical Examiner's office in Cleveland.

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Tissue

Samples from the frontal (Brodmann 10), temporal (Brodmann 22), parietal (Brodmann 1 and 2) and occipital cortex (Brodmann 17), putamen, cerebellum, hippocampus, brainstem, choroid plexus, area postrema, hypothalamus and pineal body were obtained at autopsy from a total of 12 subjects. Table 1 gives details of subjects grouped by age and the neuropathological diagnoses. Tissue blocks of about 1 cm^3 were fixed by immersion in buffered formalin, transferred to sucrose and cut into coronal sections with the cryostat as described previously [10, 13].

Immunostaining

Coronal sections of 25-μm thickness were immunostained using the avidin-biotin complex (ABC) method of Hsu et al. [9]. Tissue sections were incubated free floating overnight at 4 °C with the mAb QBEND/10 or other specified antibodies. To test specificity of immunostaining, control sections were incubated with either irrelevant antibodies, in ascites or in the absence of primary antibody. In some cases, one section was counterstained with hematoxylin and eosin (H&E). Previously immunostained or unstained adjacent tissue sections from each case were also stained with thioflavin S to reveal neurofibrillary pathology.

Immunoblotting

Immunoblots of cerebral microvessel and cerebral cortical proteins were obtained as described previously [11, 13]. Briefly, tissue preparations obtained from the occipital cortex [11] were solubilized in Laemmli sample buffer containing 3 % sodium dodecyl sulfate (SDS), and electrophoresed on 5 %–10 % polyacrylamide gels. The separated proteins on gels were then transferred to Immobilon-P membranes and probed with the mAb QBEND/10, Factor VIII or other irrelevant antibodies. Immunoreactivity was detected using the ECL Western blotting detection system kit purchased from Amersham Inc., instead of the conventional peroxidase-diaminobenzidine method. To test the localization of CD34 in the basement membrane, microvessel preparations were treated with 1 % collagenase (Boehringer #602426) for 1 h at 37 °C before solubilization and loading on gels.

Results and discussion

The specificity of the mAb QBEND/10 was confirmed by immunblotting and control immunostaining experiments. Immunblotting results showed that the antibody recognized a protein(s) of 90–100 kDa in a specific manner (Fig. 1). To prove specificity, we immunoblotted cerebral cortical proteins which were negative to CD34 mAb (Fig. 1). Both microvessel and cortical proteins probed with the primary antibody also revealed immunoreactive bands of low molecular mass (Fig. 1) that were evident even when blots were incubated with ascites medium alone or in the absence of primary antibody. Thus, these low molecular mass bands were deemed to be nonspecific, resulting from the secondary antibody (Fig. 1). Further evidence for specificity was obtained by re-probing the blots with an antibody to transferrin, which showed characteristic immunoreactivity to an 80-kDa protein (not shown). These results are in agreement with the recent observations by Watt et al. ([19], see also [3]) on the detection of 100- to 120-kDa protein in membranes from human hemopoietic progenitor cells, KG1a. CD34 is thought to be a receptor similar to other leukocyte-associated antigens expressed in myeloid and lymphoblastic leukemias [3]. Its expression in hemopoietic progenitor cells and the vascular endothelium is thought to be linked to the common origin of these cells during early embryogenesis [3, 18].

Immunostaining of normal brain tissue sections with the antibody revealed intense staining of all microvasculature (Fig. 2). Such staining of the cerebral vasculature at low or high power by light microscopy was indistinguishable from that obtained with antibody to collagen IV and similar to Factor VIII [13]. Characteristic differential density in staining between grey (Fig. 2) and white matter (not shown) and between adult and infant cortex was evident (Fig. 2a,b). Regional staining

Table 1. Age, neuropathological diagnosis and vascular anomalies detected by CD34

Case no.	Age (years)	Sex M/F	PMI (h)	Histopathological C/AD;	SP	NFT	Diagnosis CAA	Presence of abnormalities	Remarks
1	0.3	M	6	C	–	–	–	–	Low density
2	48	M	6	C	–	–	–	–	–
3	50	M	3	C	–	–	–	–	–
4	68	F	4	C	+	+	–	+	–
5	69	M	5	AD	+++	++	+	++	SP stained
7	69	F	6	AD	+++	++	++	+++	SP stained
8	72	F	6	AD	+++	++	+++	+++	SP stained
9	72	F	4	AD	++	++	+	++	–
10	79	F	4	AD	+++	+++	+++	+++	SP stained
11	83	M	12	C	+	–	–	–	–
12	90	F	5	AD	++	++	++	na	–
13	92	M	5	AD	++	++	+	na	–
14	95	M	7	AD	+++	++	++	+++	EAM in AP

Causes of death included bronchopneumonia (90 % of cases), congestive heart failure, internal hemorrhage and sudden death (infant). PMI, postmortem interval; SP, senile plaques; NFT, neurofibrillary tangels; CAA, cerebral amyloid angiopathy; C, control; AD, Alzheimer's disease; EAM, endothelial abluminal microprocesses

+ to +++ denotes increasing frequency. Staining was assessed in frontal, temporal, entorhinel and occipital cortices and hippocampus, putamen, cerebellum, area postrema (AP), median eminence and the pineal

na, Not assessed, in these cases only choroid plexus and AP were examined

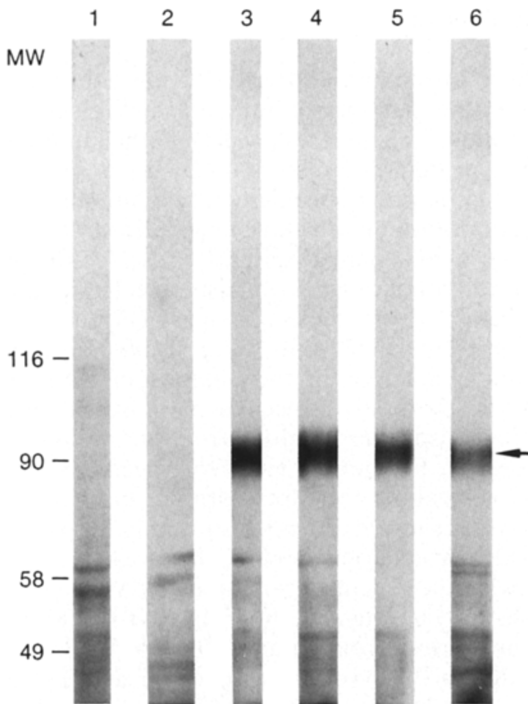

Fig. 1. Immunoblots of cerebral microvessel proteins recognized by QBEND/10 monoclonal antibody (mAb) to CD34. SDS solubilized preparations of microvessels (*lanes 1, 3–6*) and cerebral cortex (*lane 2*) from Alzheimer's disease (AD) and age-matched control subjects were electrophoresed, transferred to Immobilon-P membranes and immunoreacted with ascites medium (*lanes 1*) or mAb QBEND/10 (*lanes 2–6*). QBEND/10 showed specific antigenicity to 90–100 kDa protein(s) (*arrow*) evident in microvessels only but not in cortex. *Lanes 3* and *4* from two AD subjects and *lanes 5* and *6* from two controls. The lower molecular mass (MW) bands are nonspecific (see text)

patterns in the gray matter were similar with the most intense in neocortex. In accord with the findings of Schlingemann et al. [18], we could not make a clear distinction in staining between the luminal and abluminal sides of vessels (Fig. 2) at the light microscope level in either controls or AD subjects. There was no evident staining of the glia limitans. Among larger vessels arterioles were stained and both adventitia and intima appeared equally stained (Fig. 2d). The basement around vascular smooth muscle cells was also stained. In neurologically normal subjects extensive analysis of different brain regions showed no evidence of staining in any other structure besides cerebral vessels. The capillaries of the choroid plexus were also stained (Fig. 2e) but, as with collagen IV, the epithelial cells of the choroid were negative. However, there was weak staining of the basement membrane of choroidal epithelial cells. Clear immunostaining was also evident in regions with fenestrated capillaries and devoid of tight junctions *vis a vis* the non blood-brain barrier areas including the area postrema, median eminence and the pineal (Fig. 2f). Our observations here extend and confirm prior studies in which staining was reported to be restricted to the capillary endothelium in cerebral cortex and the pituitary from normal subjects [19].

In AD, vascular density appeared to be markedly reduced as exhibited by shorter discontinuous vessel profiles and less extensive capillary network (Fig. 3a,c,f. Fig. 2a), Although consistent with previous observations [1, 6, 17], this phenomena presumably occurs due to the development of amyloid plaques, which may tend to displace the vasculature [15] but may not necessarily imply substantial changes in vascular capacity [1]. Only in cases with marked pathology could structures resembling vascular sprouts or EAM be readily seen (Fig. 3b–f). In a number of cases structures resembling amyloid plaques near vessels were also stained (Fig. 3g) suggesting that some plaques may contain CD34 epitopes or vascular proteins. In most AD cases, stained vessels in both gray and white matter exhibited looping, "kinking" [6] and extensive tortuosity (Fig. 3h). There was no evidence of staining in any other cellular elements including microglia that are abundant in AD tissue [16]. To determine whether the localization of CD34 was exclusively associated with endothelial cells in cerebral microvessels from AD subjects and chemically dissect the endothelium from the basement membrane, we treated microvessel preparations with collagenase prior to immunoblotting (Fig. 4). Previous studies have used collagenase preparations to remove the basement membrane [20]. Surprisingly, the results showed that CD34 immunoreactivity was reduced, whereas Factor VIII known to be localized to endothelial cells remained unaffected in the collagenase-treated preparations compared to untreated vessels (Fig. 4). While these results are contrary to those of Schlingemann et al. [18] suggesting localization of CD34 to the endothelium *per se*, they suggest that perhaps some of the CD34 reactive elements are associated with the basement membrane. It is possible that removal of the basement membrane by collagenase affects CD34 immunostained EAM and fibrils supported by it on the abluminal side [18] of cerebral microvessels from AD subjects.

Our observations on the presence of vascular anomalies or EAM (Fig. 3) are consistent with the increased proportion of 'strings' or 'streamers' present in brain vessels of subjects with AD ([16] and Kalaria, unpublished observations) that were stained by anti-collagen sera. Whether these peculiarities represent collapsed, degenerating or proliferating vessels is uncertain. However, we have also found chemical changes in the basement membrane; hydroxyproline, the main constituent of collagen IV, is increased in isolated cerebral microvessels from subjects with AD compared to age-

Fig. 2a–f. Immunostaining of the cerebral microvasculature in neurologically normal subjects. QBEND/10 mAb recognized capillaries in all brain regions including regions with fenestrated capillaries. **a,b** Neocortical vascular staining in aging control (case 4) and infant (case 1). **c** Putamen with large neurons in adult control. **d** Large stained vessel. **e** Choroid plexus and **f** pineal, similar staining was seen in the area postrema. Intense vascular staining by anti-collagen IV serum was seen in adjacent sections of above. *a–c,e,f* H&E counterstaining, × 250; **d** × 500

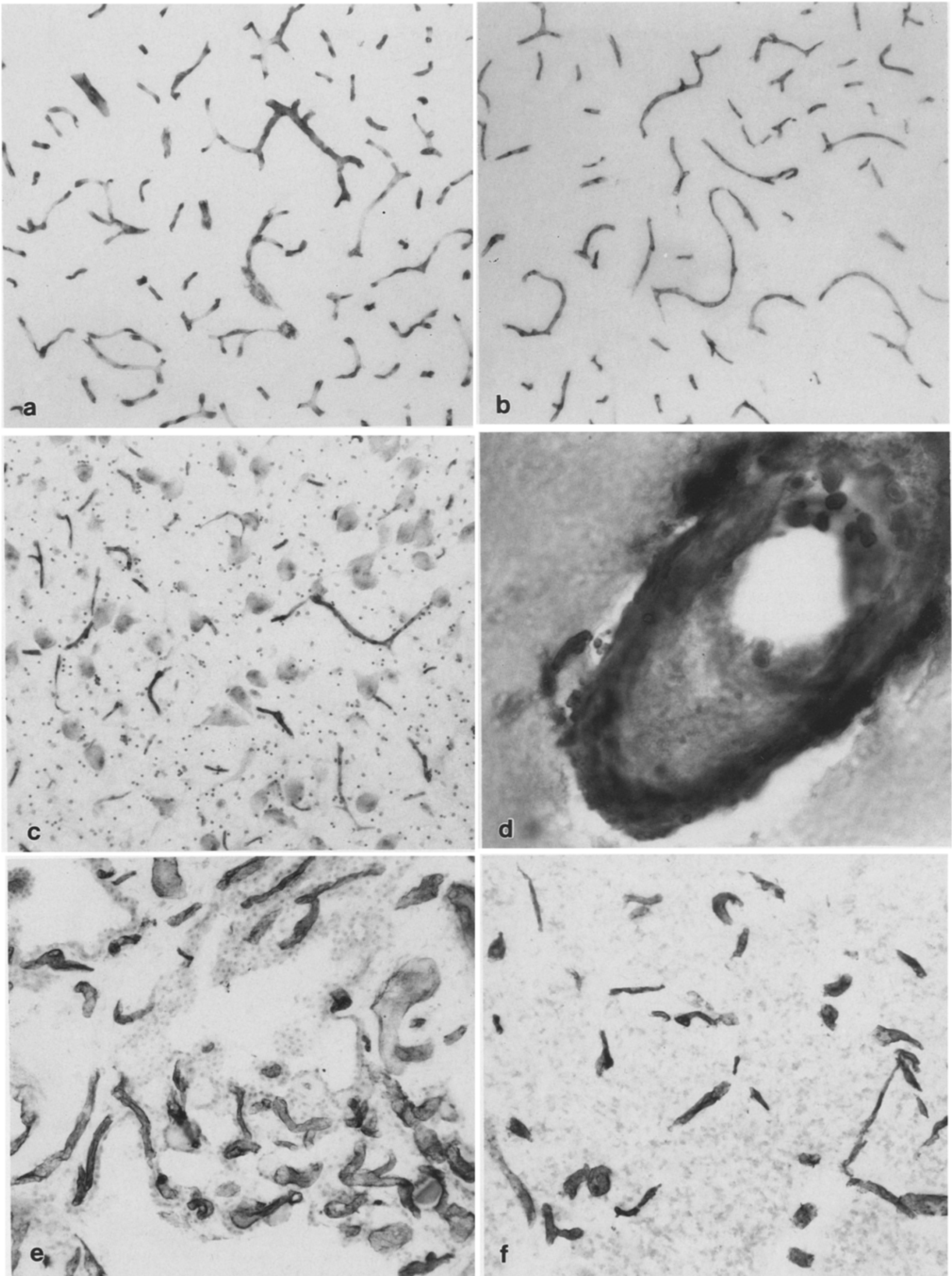

Fig. 2a–f

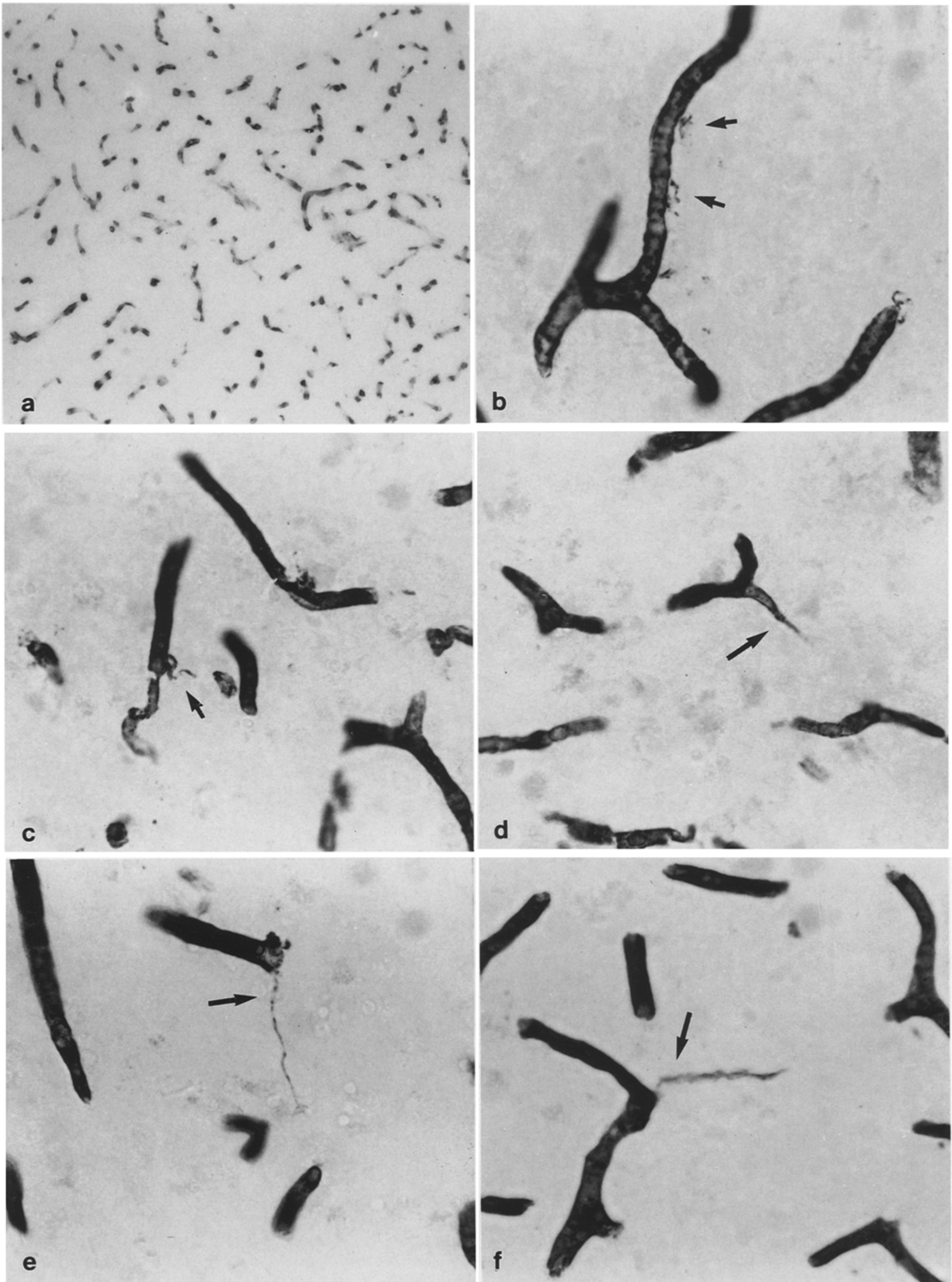

Fig. 3a–f

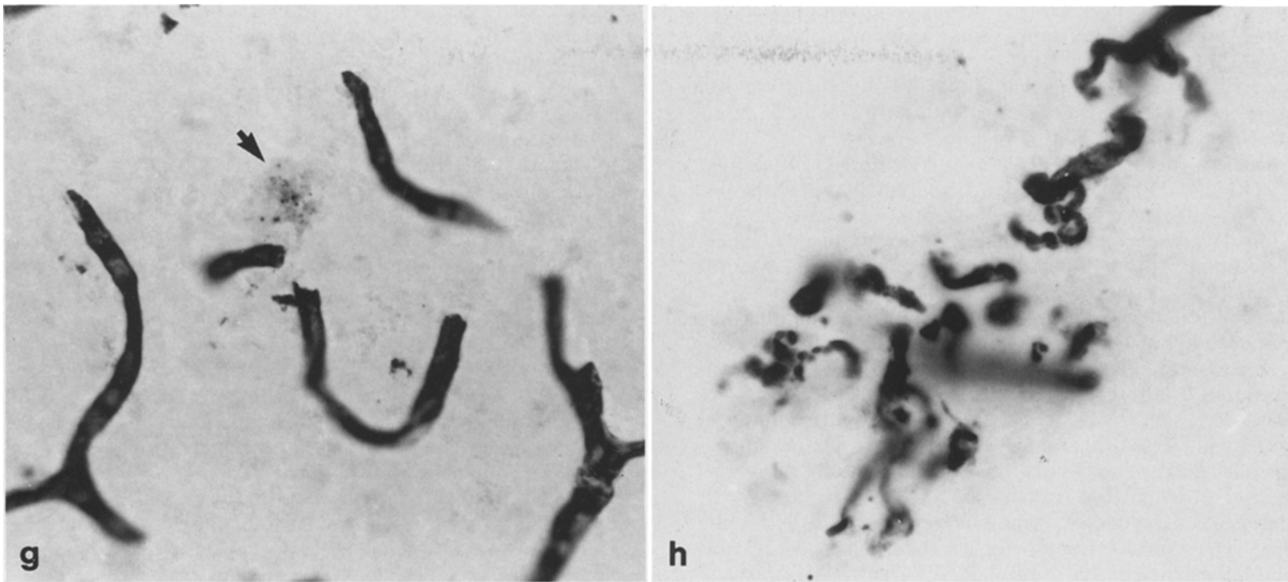

Fig. 3a–h. Immunostaining of the cerebral microvasculature in AD subjects. QBEND/10 mAb recognized capillaries with vascular sprouts and endothelial abluminal microprocesses (EAM). **a** Neocortical vascular staining in AD subject (case 7). **b–f** Examples of EAM (*short arrows*) and vascular sprouts (*longer arrows*) in neocortex of AD subjects. **g** Plaque-like structure near stained vessels. **h** "Kinking" vessels [6] in white matter from AD subject (case 8). **a** × 250; **b–h** × 500

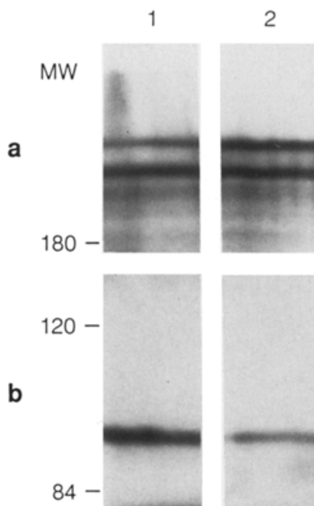

Fig. 4a,b. Immunoblots of cerebral microvessels showing the effect of collagenase treatment on CD34 immunoreactivity. Untreated (*lane 1*) and collagenase-treated (*lane 2*) cerebral microvessels from a 65-year-old AD subject were transferred to Immobilon-P membrånes and immunoreacted with antibody to Factor VIII (**a**) or mAb CD34 (**b**). **a** shows unaffected immunoreactivity to Factor VIII [*double band,* molecular mass (MW) about 200 kDa] and **b** shows markedly reduced immunoreactivity to CD34 after treatment with collagenase (*lane 2*) to remove basement membrane. Molecular mass markers shown in kDa

matched controls [12]. We propose that our observations with QBEND/10 mAb in AD and possibly other neurodegenerative diseases show clear anomalies in the cerebral endothelium. These may result from reactive responses to maintain a balance between the endothelium and basement membrane and compensate for changes in the vascularity during the pathogenetic process. It is possible that growth factors or mediators released from reactive astrocytes [4, 5], macrophages or microglia that proliferate during disease may induce these vascular changes. However, these findings of vascular reactivity are not incompatible with evidence for reactive trophic influences on neurites to cause abnormal sprouting in the hippocampus of AD subjects [7, 8].

In summary, we describe the expression of the leukocyte antigen CD34 by the human brain endothelium. All microvasculature including those of the circumventricular organs were immunostained by the CD34 antibody. In AD, there were an increased number of vessels with vascular anomalies and EAM most apparent in subjects with marked cortical pathology as indicated by the presence of numerous plaques and tangles.

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